TIMP-3 inhibits the procollagen N-proteinase ADAMTS-2

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ADAMTS-2 is an extracellular metalloproteinase responsible for cleaving the N-propeptides of procollagens I–III; an activity necessary for the formation of collagenous ECM (extracellular matrix). The four TIMPs (tissue inhibitors of metalloproteinases) regulate the activities of matrix metalloproteinases, which are involved in degrading ECM components. Here we delineate the abilities of the TIMPs to affect biosynthetic processing of procollagens. TIMP-1, -2 and -4 show no inhibitory activity towards ADAMTS-2, in addition none of the TIMPs showed inhibitory activity towards bone morphogenetic protein 1, which

is responsible for cleaving procollagen C-propeptides. In contrast, TIMP-3 is demonstrated to inhibit ADAMTS-2 *in vitro* with apparent K_i values of 160 and 602 nM, in the presence of heparin or without respectively; and TIMP-3 is shown to inhibit procollagen processing by cells.

Key words: ADAMTS-2, bone morphogenetic protein 1 (BMP1), extracellular matrix, metalloproteinase, procollagen, tissue inhibitor of metalloproteinase (TIMP).

INTRODUCTION

The major fibrillar collagens, which constitute the major fibrous components of vertebrate ECM (extracellular matrix), are synthesized as procollagens, with N- and C-propeptides that must be cleaved to produce mature monomers capable of assembling into fibrils [1–3]. ADAMTS-2, an extracellular ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) metalloproteinase, is capable of providing the pNP (procollagen N-proteinase) activity that cleaves the N-propeptides of procollagens I–III *in vitro*. Mutations in the *ADAMTS-2* gene in dermatosparaxis and Ehlers–Danlos syndrome VIIC demonstrate that this enzyme is responsible for biosynthetic processing of procollagens I and III N-propeptides *in vivo* [4–6]. BMP (bone morphogenetic protein) 1, a member of the astacin subfamily of metalloproteinases, is capable of providing the pCP (procollagen C-proteinase) activity that removes the major procollagen C-propeptides *in vitro* and *in vivo* [7–9]. However, despite the importance of BMP1 and ADAMTS-2 to collagen biology, only one endogenous inhibitor has recently been found for BMP1-like proteinases [10] and only the *Drosophila* protein papilin has been demonstrated to be capable of inhibiting mammalian ADAMTS-2 [11].

TIMPs (tissue inhibitors of matrix metalloproteinases) constitute a family of four structurally related metalloproteinase inhibitors in vertebrates [12]. TIMPs are best characterized as inhibitors of the MMPs (matrix metalloproteinases), also known as matrixins, with which they bind with a 1:1 stoichiometry to effect inhibition [13]. All TIMPs have inhibitory activity towards all MMPs, although TIMP-1 is a relatively poor inhibitor of MMP-19 and of some MT (membrane type)-MMPs [14]. In addition, TIMP-3 differs from the other TIMPs in inhibiting certain non-MMP metalloproteinases, including ADAM-10, ADAM-12, ADAM-17 and the aggrecanases ADAMTS-4 and -5 [15–18]. Inhibitory activity towards ADAM-10 has also been reported for TIMP-1 [15].

Here we demonstrate that TIMP-3 inhibits the pNP ADAMTS-2 with apparent K_i values of 160 and 602 nM in the presence of

heparin or without respectively. It can also inhibit the processing of procollagen I by cells. This is the first report of an endogenous inhibitor for ADAMTS-2 or any pNP. In addition, none of the TIMPs tested showed inhibitory activity towards BMP1.

EXPERIMENTAL

Materials

Human TIMP-1, -2, -3 and -4 (with IC_{50} values of ~3, 2.5, 2.5 and 2 nM against MMP-2 respectively) and anti-TIMP-3 antibody were obtained from R&D Systems. Anti-(protein C) antibody beads were purchased from Roche Applied Science. Anti-(protein C)–peroxidase was purchased from Boehringer Mannheim. N-TIMP-3, the active N-terminal domain of TIMP-3 was expressed at high yield in *Escherichia coli* and refolded from inclusion bodies as described previously [18]. N-TIMP-3 was used instead of commercial full-length TIMP-3 for some experiments, as the former is much more economical and has inhibitory activity comparable to that of the full-length protein [18]. pcDNA4/TO, T-RexTM-293 cells, LipofectamineTM, Zeocin and tetracycline were purchased from Invitrogen. All proteinase inhibitors were from Sigma.

Production of recombinant proteins

FLAG-tagged human recombinant ADAMTS-2 and BMP1 (both used in inhibition assays) were produced and purified as described previously [6, 19]. To produce protein C-tagged ADAMTS-2 (used in pull-down assays), sequences encoding EDQVDPRLIDGK followed by a stop codon were added by PCR to the 3'-end of a human ADAMTS-2 cDNA insert described previously [6], corresponding to full-length human ADAMTS-2 without its signal peptide sequence and ligated between the NheI and EcoRI sites of vector pcDNA4/TO/BM40s. The resulting construct expresses full-length ADAMTS-2, differing from native protein only in replacement of the native signal peptide by the BM40 signal peptide and by the addition of a C-terminal protein C

Abbreviations used: ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; BMP, bone morphogenetic protein; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; MEF, mouse embryonic fibroblast; MMP, matrix metalloproteinase; N-TIMP-3, N-terminal domain of tissue inhibitor of metalloproteinase-3; pNP, procollagen N-proteinase; pCP, procollagen C-proteinase; TIMP, tissue inhibitors of metalloproteinase.

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epitope tag. T-RexTM-293 cells were maintained as described [20] and were transfected at 80% confluence with 10 µg expression vector per 100-mm diameter culture dish using LipofectamineTM. After 36 h, cells were selected with 200 µg/ml Zeocin and resistant clones were isolated. Culture media were analysed by immunoblot and the clone expressing the highest levels of ADAMTS-2 upon induction with 1 µg/ml tetracycline was used for production of protein C-tagged ADAMTS-2.

To prepare purified protein C-tagged ADAMTS-2, cells grown to confluence were washed once with PBS and switched to serum-free DMEM (Dulbecco's modified Eagle's medium) containing 40 µg/ml soybean trypsin inhibitor and 1 µg/ml tetracycline. Conditioned medium was harvested 24 h later and protease inhibitors were added to final concentrations of 0.2 mM PMSF, 1 mM *N*-ethylmaleimide and 1 mM *p*-aminobenzoic acid. Harvested medium was centrifuged to remove cell debris and supernatants were stored at -70 °C. Protein C-tagged ADAMTS-2 was bound to 0.5 ml anti-protein C matrix and the matrix was washed with 10 ml wash buffer (20 mM Tris/HCl, pH 7.5, 1 M NaCl and 1 mM CaCl₂) following the supplier's protocols. ADAMTS-2 was eluted by 20 mM Tris/HCl, pH 7.5, 0.1 M NaCl and 5 mM EDTA, and fractions were collected and analysed by immunoblot analysis.

Inhibition assays

Procollagen substrates, metabolically radiolabelled with 2,3-[³H]proline, were prepared and purified as described previously [19]. Purified ADAMTS-2 or BMP1 were pre-incubated for 30 min at 37 °C with TIMP-1, -2, -3, -4 or N-TIMP-3. For Figure 3(A), N-TIMP-3 and ADAMTS-2 were pre-incubated with 100 µg/ml heparin. Procollagen I, II or III (210 ng) was then added and assays were performed for 20 h at 37 °C in 50 mM Tris/HCl, pH 7.5, 150 mM NaCl and 5 mM CaCl₂. Reactions were stopped by the addition of 10× SDS/PAGE loading buffer and boiling for 5 min. Samples were subjected to SDS/PAGE on 5% acrylamide gels, which were subsequently treated with EN³HANCE (DuPont) and exposed to X-ray film. Relative densities of collagen bands on autoradiograms were measured by scanning densitometry using NIH Image 1.62 software and apparent inhibition constants (apparent K_i values) were calculated using GraphPad Prism 4 software. The rate of procollagen processing was taken as proportional to the amount of active ADAMTS-2 remaining uncomplexed to TIMP-3. Thus, after preincubation with TIMP-3, the concentration of free active ADAMTS-2 ([E]) can be calculated using the following equation:

$$[E] = [E_t]/(1 + [TIMP]/K_i) \quad (1)$$

where $[E_t]$ is the total ADAMTS-2 used in the assay and K_i is the apparent inhibition constant between TIMP-3 and ADAMTS-2.

The procollagen processing activity of ADAMTS-2, under ideal conditions, can be expressed as:

$$V = k_1[E][S]/K_m \quad (2)$$

where K_m is the apparent dissociation constant between procollagen and ADAMTS-2 and k_1 is the turnover constant of procollagen processing by ADAMTS-2. The equation can be expressed as:

$$\begin{aligned} V &= k_1[S]([E_t]/(1 + [TIMP]/K_i))/K_m \\ &= (k_1[E_t][S]/K_m)/(1 + [TIMP]/K_i) \\ &= V_0/(1 + [TIMP]/K_i) \end{aligned} \quad (3)$$

where V_0 is the ADAMTS-2 activity in the absence of TIMP-3.

Co-immunoprecipitations and immunoblots

Indicated amounts of ADAMTS-2 and TIMP-3 were co-incubated and then immunoprecipitated with anti-TIMP-3 antibodies, using conditions described previously [21,22]. Subsequently, samples were analysed by immunoblot using 1:5000 dilutions of anti-TIMP-3 and anti-(protein C) antibodies. Immunoblots involved electrotransfer to Immobilon-P membranes (Millipore), blocking in PBS with 2% (w/v) BSA and 0.05% Tween 20, and washes were all as described previously [6]. Blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit antibody diluted 1:5000 (Amersham Biosciences). Washed blots were incubated for 4 min in SuperSignal West Pico substrate (Pierce) and exposed to X-ray film.

Culturing of MEFs (mouse embryonic fibroblasts)

Immortalized MEFs, prepared and cultured as described previously [23], were treated with dextran sulfate to augment efficiency of enzymatic conversion of procollagens to collagens, essentially as described [6]. Briefly, MEFs were grown to ~80% confluence and treated overnight in DMEM and 10% (v/v) foetal bovine serum containing 50 µg/ml ascorbate. The following day, cells were switched to serum-free medium containing amounts of N-TIMP-3 as indicated in the text, 50 µg/ml ascorbate, 40 µg/ml soybean trypsin inhibitor and 0.01% dextran sulfate (Amersham). After 20 h, cell layers were rinsed twice with PBS and scraped into hot SDS/PAGE loading buffer. After SDS/PAGE, electrotransfer and washing, as described above, blots were probed with antibody LF-67 [24] (a gift from Larry Fisher, National Institute of Dental Research, Bethesda, MD, U.S.A.), directed against sequences in the pro- $\alpha 1$ (I) C-telopeptide region and diluted 1:5000. Blocking was for 1 h with 5% (w/v) milk powder in PBS and secondary antibodies were as described above.

RESULTS

TIMP-3 binds and inhibits ADAMTS-2

As TIMP-3 is known to inhibit several ADAM/ADAMTS proteinases [15–18], we tested its ability to inhibit the cleavage of the type II procollagen N-propeptide by ADAMTS-2. Under the assay conditions, ADAMTS-2 almost completely processed procollagen II to the pC $\alpha 1$ (II) processing intermediate, which retains the C-propeptide, but from which the N-propeptide has been removed (Figure 1A). However, TIMP-3 was able to inhibit this activity in a dose-dependent manner. Recombinant N-TIMP-3 was also effective in inhibiting ADAMTS-2 pNP activity against procollagen types I and III (Figure 1B). TIMP-1, -2 or -4 did not show any inhibitory effect towards ADAMTS-2 pNP activity (results not shown).

TIMP inhibition of MMPs involves formation of a tight complex between the two types of proteins [12]. To determine whether ADAMTS-2 inhibition by TIMP-3 similarly involves formation of a stable complex, TIMP-3 and ADAMTS-2 were co-incubated, followed by immunoprecipitation by anti-TIMP-3 antibody. Shown here (Figure 1C) and described previously [6], 132 and 118 kDa bands, which represent the full-length mature and truncated versions of secreted ADAMTS-2, were co-precipitated with TIMP-3 indicating complex formation. Co-precipitation is increasingly evident at TIMP-3/ADAMTS-2 ratios greater than 1:1, as a greater percentage of input ADAMTS-2 is pulled down from solution by available TIMP-3.

In contrast with its ability to inhibit the pNP activity of ADAMTS-2, TIMP-3 had no inhibitory activity, even at 50-fold excess, towards the pCP activity of BMP1, which is responsible

Figure 2 N-TIMP-3 inhibits ADAMTS-2 with an apparent K_i of 602 nM

(A) A constant amount of ADAMTS-2 (12 nM) was incubated with increasing amounts of N-TIMP-3 (0–1000 nM), prior to incubation with ^3H -labelled type II procollagen, followed by SDS/PAGE and autofluorography. A Table of the percentage procollagen II processed at each concentration of N-TIMP-3 is shown below the autofluorogram. Data were plotted and an apparent K_i of 618 nM was calculated using GraphPad Prism 4 software. The K_i range was 411–826 nM and the standard error was 80.7 nM (95.5 confidence intervals). **(B)** A constant amount of ADAMTS-2 (4 nM) was incubated with increasing amounts of N-TIMP-3 (0–500 nM), prior to incubation with ^3H -labelled type II procollagen, followed by SDS/PAGE and autofluorography. A Table of the percentage of procollagen II processed at each concentration of N-TIMP-3 is shown below the autofluorogram. Data were plotted and an apparent K_i of 602 nM was calculated using GraphPad Prism 4 software. The K_i range was 396–809 nM and the standard error was 84.4 nM (95.5 confidence intervals).

ADAMTS-2 processed only 56 % of the substrate in the absence of TIMP-3 (Figure 2B), ensuring that all TIMP-3 inhibitory activity would be detected. Under these conditions, the apparent K_i was calculated to be 602 nM. Agreement of apparent K_i values calculated from the different assay conditions of Figures 2(A and B) supports the likelihood of their accuracy.

It has been shown previously that TIMP-3, unlike the other TIMPs, binds to extracellular sulfated glycosaminoglycans [25] and that heparin and heparan sulfate can increase the association rate of TIMP-3 with MMP-2 [26]. Thus, we examined the possibility that heparin might affect the inhibition of ADAMTS-2 pNP activity by TIMP-3. To this end, a fixed amount of

Figure 3 N-TIMP-3 inhibits ADAMTS-2 with an apparent K_i of 160 nM in the presence of heparin

(A) A constant amount of ADAMTS-2 (12 nM) was incubated with a constant amount of N-TIMP-3 (200 nM) and with increasing amounts of heparin, prior to incubation with ^3H -labelled type II procollagen, followed by SDS/PAGE and autoradiography. The amount of heparin used is plotted against the ratio of procollagen II compared with the pC α 1(I)-form (which retains the C-propeptide, while the N-propeptide has been removed). (B) A constant amount of ADAMTS-2 (12 nM) was incubated with increasing amounts of N-TIMP-3 (0–1000 nM) and 100 $\mu\text{g}/\text{ml}$ heparin, prior to incubation with ^3H -labelled type II procollagen, followed by SDS/PAGE and autoradiography. A Table of the percentage of procollagen II processed at each concentration of N-TIMP-3 is shown below the autoradiogram. Data were plotted and an apparent K_i of 160 nM was calculated using GraphPad Prism 4 software. The K_i range was 11.4 to 309.3 nM and the standard error was 60.9 nM (95 % confidence intervals).

ADAMTS-2 and TIMP-3 were incubated in the presence of increasing amounts of heparin. Although heparin alone had no effect on ADAMTS-2 pNP activity (results not shown), heparin had a marked effect in enhancing the ability of TIMP-3 to inhibit ADAMTS-2 pNP activity, where 100 $\mu\text{g}/\text{ml}$ of heparin was the most efficacious concentration for enhancement of TIMP-3 inhibition of ADAMTS-2 (Figure 3A). Thus, to determine the apparent K_i of TIMP-3 inhibition of ADAMTS-2 in the presence of heparin, a fixed amount of ADAMTS-2 was incubated with increasing amounts of N-TIMP-3 in the presence of 100 $\mu\text{g}/\text{ml}$ heparin, prior to an assay for cleavage of type II procollagen. As shown in Figure 3(B), heparin markedly increased ADAMTS-2 inhibition, so that the apparent K_i was calculated to be 160 nM. The bell-shaped curve of Figure 3(A) suggests a model that, at optimum concentration, a single heparin molecule binds to molecules of both ADAMTS-2 and TIMP-3, thus increasing

Figure 4 N-TIMP-3 inhibits the pNP activity of cells

N-TIMP-3 was added to the culture media of MEF cells, to a final concentration of 250 or 800 nM, in the presence of 0.01 % dextran sulfate. Following SDS/PAGE and electrotransfer, pro- α 1(I)-derived chains in cell layer samples were probed using an antibody directed against sequences in the pro- α 1(I) C-telopeptide domain.

interactions between the two proteins, whereas at higher heparin concentrations individual ADAMTS-2 and TIMP-3 molecules are more likely to be bound to separate heparin molecules, so that interactions of the two proteins are no greater than in the absence of heparin.

TIMP-3 inhibits procollagen processing by cells

We next sought to determine whether TIMP-3 was capable of inhibiting the pNP activity of cells. As shown in Figure 4, type I collagen was almost entirely processed to the mature form in the absence of any type of experimental treatment of MEFs. In contrast, addition of N-TIMP-3 to MEF cultures inhibited cleavage of the N-propeptide in a dose-dependent manner, so that markedly greater amounts of pN α 1(I) chains (forms that retain the N-, but not the C-propeptide) and markedly reduced amounts of mature α 1(I) chains were observed. Unprocessed pro- α 1(I) chains and pC α 1(I) chains, which are not normally incorporated into cell-associated ECM in culture [27], were not observed in MEF cell layers under any of the conditions used.

DISCUSSION

Here we have identified the first natural inhibitor of the pNP ADAMTS-2. It should be noted that two other known pNPs exist, ADAMTS-3 and ADAMTS-14 [28,29], and that these proteinases probably provide residual pNP activity noted in dermatosparaxis and Ehlers–Danlos syndrome type VIIC tissues, which are devoid of ADAMTS-2. However, it is likely that inhibitors of ADAMTS-2 would have similar effects on ADAMTS-3 and -14, as ADAMTS-2, -3 and -14 form a subgroup within the ADAMTS proteinases, sharing an identical protein domain structure and high sequence similarity in their mature active regions, which are located downstream of the prodomain [30].

Although the apparent K_i for N-TIMP-3 inhibition of ADAMTS-2 was estimated to be 602 nM in solution, this inhibition was improved to an apparent K_i of 160 nM in the presence of heparin. If the mechanism where heparin enhances the inhibition of ADAMTS-2 by N-TIMP-3 is similar to that where it enhances MMP-2 inhibition by TIMP-3 [26], then heparin acts by binding both ADAMTS-2 and N-TIMP-3, thereby increasing the local concentration of the two reactants and increasing their rate of interaction. With regard to this possibility, both ADAMTS-2 and TIMP-3 have been demonstrated previously to bind heparin with strong affinity, and N-TIMP-3 was found to bind heparin with an affinity similar to that of full-length TIMP-3 [6,25]. Both ADAMTS-2 and TIMP-3 are normally bound to sulfated glycosaminoglycans associated with ECM and cell surfaces [6,25]. Such localization of TIMP-3 to the pericellular space is likely to aid its ability to inhibit cell surface sheddases [25],

whereas localization of ADAMTS-2 to the same space is likely to aid its role in procollagen biosynthetic processing, which is thought to occur at or near the cell surface [30]. Co-localization to the same space by the same types of molecules suggests that, as with heparin, sulfated glycosaminoglycans associated with ECM and/or cell surfaces increase the local concentrations of both ADAMTS-2 and TIMP-3 in the same space, thereby increasing their rate of interaction and, in this way, adding to the regulation of collagen biosynthesis. Thus, it is probable that the apparent K_i obtained in the presence of heparin is more representative of ADAMTS-2/TIMP-3 interactions *in vivo*, than is the apparent K_i obtained in the absence of heparin.

TIMP-3 has been shown to inhibit certain ADAM/ADAMTS proteinases, while TIMP-1 has been shown to inhibit ADAMTS-10 [13,14]. Here, the apparent K_i of 160 nM shows TIMP-3 to be an effective ADAMTS-2 inhibitor in the presence of heparin and it is also capable of inhibiting the pNP activity of fibrogenic cells. Thus, the result of effective inhibition of ADAMTS-2 and the other pNPs, namely ADAMTS-3 and -14, would be collagen monomers that retain N-propeptides. Importantly, monomers which retain N-propeptides have been shown to form fibrils that are thin, abnormally cross-linked and likely to have rapid metabolic turnover [31]. Thus, inhibition of ADAMTS pNPs by TIMP-3 could result in interference with formation of an irreversibly stable collagenous ECM, thereby providing a previously unknown level of regulation of net ECM deposition.

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